LIVER MICROSOMAL HYDROXYLATING SYSTEM IN MICE WITH THE TOXIC FORM OF INFLUENZA

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A leading factor in the pathogenesis of acute viral infections is induction of a combination of specific and nonspecific reactions, leading to the accumulation of physiologically active metabolites in the tissues, a change in the state of the system of immunochemical homeostasis, and the development of toxicosis [2, 8, 9, 11]. In particular it has been shown that several biochemical changes in the blood of animals and man in an acute form of influenzal infection are due to activation of a free-radical process, accompanied by an increase in the methemoglobin concentration and production of a nitrogen oxide ($NO^{\cdot}$) [3]. Considering the cytotoxic action of products of free-radical reactions, the function of molecular detoxication mechanisms in the tissues and organs of infected animals and, in particular, the efficacy of the microsomal hydroxylating system of the liver are particularly important.

It is generally considered that the microsomal hydroxylating system of the liver consists of three main components: NADPH-cytochrome P-450 reductase, cytochrome P-450, and phospholipids of the matrix of the microsomal membranes [7]. Accordingly, the following parameters were chosen as criteria for assessing the functional state of the microsomal hydroxylating system: 1) the level of the catalytically active form of cytochrome P-450; 2) the efficacy of peroxide O-dealkylation of esters, catalyzed by cytochrome P-450 [10]; 3) the concentration of lipid peroxidation (LPO) products in the matrix of these membranes and in the lipid microenvironment of the proteins; 4) the structural organization of the lipid matrix of the membranes [7].

EXPERIMENTAL METHOD

Four series of experiments were undertaken on male CBA mice. In each series there were three groups of animals, with 10 mice in each group: 1) control; 2) animals infected with an apathogenic strain of virus; 3) animals infected with a pathogenic strain of the virus. Influenza virus A/Victoria/35/72 (H3N2), adapted (pathogenicity $4.2 \cdot \log LD_{50}$) and not adapted (apathogenic) to mice were used. The peak of virus reproduction in the lungs in response to injection of the pathogenic strain was observed after 48 h and reached a level of $8.3 \cdot \log EID_{50}$. Death of the animals was observed on the 6th day after infection. The microsomal fraction was isolated from mouse liver homogenate and activity of p-nitroanisole-O-demethylase in the liver homogenate was determined by the method described in [12]. Membrane preparations were resuspended in 70 mM phosphate buffer, pH 7.8. The content of the catalytically active form of cytochrome P-450 in the mouse liver was determined by the EPR spectroscopy method. The content of the active (low-spin) form of cytochrome P-450 was estimated from the intensity of the characteristic line ($g = 2.26$) in the EPR spectrum of frozen tissue (77°K) [1]. EPR signals were recorded on a small spectrometer, made by the "Svetlana" Leningrad Optico-Electrical Factory, using microwave radiation with a power of 50 mV. The concentration of fluorescent LPO products in the samples was monitored and the fatty acid composition of the lipids analyzed as described in [2]. Standard mixtures of methyl esters were used in the analysis, and 2,4,6-di-*tert*-butyl

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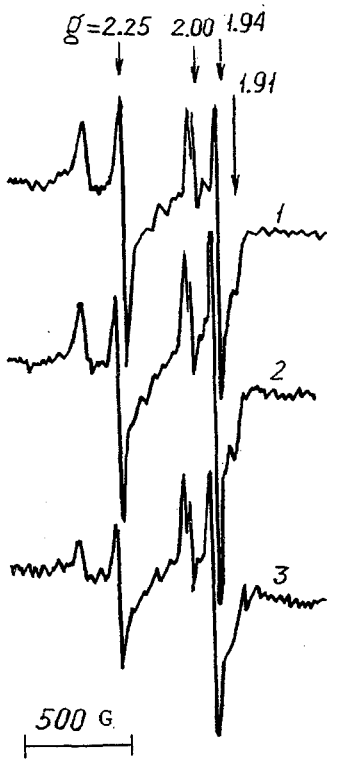

Fig. 1

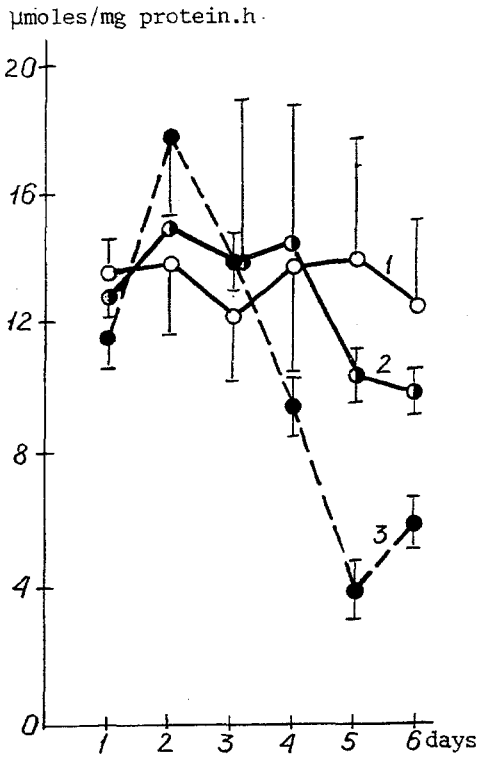

Fig. 2

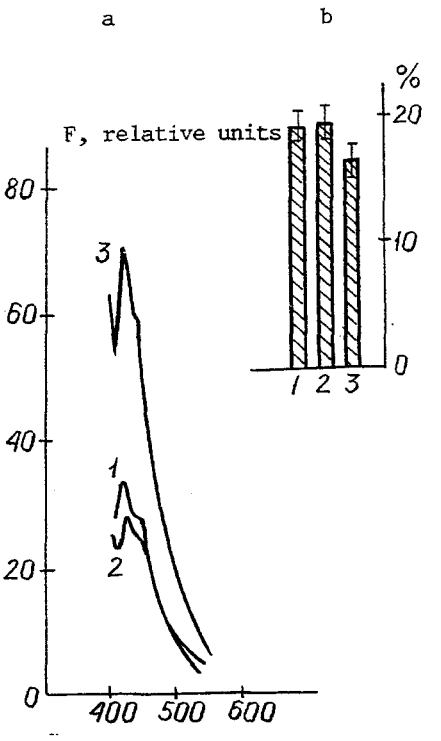

Fig. 3

Fig. 1. Results of EPR spectroscopy to assess levels of active forms of cytochrome P-450 (g = 2.25) in liver of mice: 1) control, 2) infected with apathogenic virus, 3) infected with pathogenic virus.

Fig. 2. Circadian rhythm of p-nitrophenol-O-methylase activity in mouse liver: 1) control, 2) infected with apathogenic virus, 3) infected with pathogenic virus.

Fig. 3. Changes in fatty acid composition of lipids of protein microenvironment in liver microsomes of mice: 1) control, 2) infected with apathogenic virus, 3) infected with pathogenic virus. a) content of fluorescent LPO products in fatty acid composition of phosphatidylethanolamine and phosphatidylserine, cross-linked with membrane proteins by GDA, b) relative content of palmitic acid (per cent of total fatty acid content) in fatty acid composition of phosphatidylethanolamine and phosphatidylserine, "cross-linked" with membrane proteins by GDA.

phenol (USSR origin) was used as the internal standard. The methyl esters of fatty acids (MEFA) were analyzed on a "Pye 104" gas–liquid chromatograph (England) with flame-ionization detector. To separate phosphatidylethanolamine and phosphatidylserine from the microenvironment of the proteins in the lipid matrix of the microsomal membranes, a bifunctional reagent (glutaric dialdehyde, GDA), "ligating" the amino groups of lipids and proteins, was used. GDA was purified from its polymeric forms by the method described in [4]. A suspension of microsomal membranes (1 mg protein/ml) was treated with 5 mM GDA, and this was followed by incubation at 37°C for 30 min. Lipids were extracted and the content of lipid phosphorus analyzed as in [2]. The content of α-tocopherol in the lipid extracts was determined by a fluorometric method. The sorbent was hexane, excitation occurred at 295 nm, emission at 320 nm. Measurements were made on a "Hitachi MPF-2A" spectrofluorometer (Japan). The orderliness of the acyl chains of the membrane lipids was evaluated by spin probing. The constant of superfine interaction ($2A_{//}$), calculated from the EPR spectrum of the spin probe in the membranes [6], was used as criterion of the degree of order. The spin probe, 5-doxylstearic acid (5DS), was injected in the proportion of one molecule of the probe to 200 molecules of lipids (the samples were thermostatic at 10°C). The following reagents were used: nicotinamide and glu-

cose-6-phosphate were from "Reanal" (Hungary), and the 5-DS and standards of MEFA were from "Sigma" (USA). The experimental data are given with twice the standard error (level of significance $p < 0.05$).

EXPERIMENTAL RESULTS

Liver tissue, frozen at the temperature of liquid nitrogen (77°K), and liver tissue homogenate were studied in a series of experiments.

On the 5th-6th day after infection with pathogenic influenza virus a decrease in the active form of cytochrome P-450 was observed in the animals' liver. In fact, as the data in Fig. 1 show, the intensity of the EPR signal $g = 2.25$ of the liver tissue sample with the toxic form of influenza was significantly lower than the corresponding signal in tissue from control animals and animals infected with the apathogenic virus.

The results of enzymic analysis agreed with the results of investigation of the state of the microsomal hydroxylating system obtained by EPR microscopy. For instance, by the 5th day there was a decrease in p-nitrophenyl-O-demethylase activity in the liver homogenate from mice infected with the pathogenic strain of the virus compared with activity of that enzyme in liver homogenate from control animals and from animals infected with the apathogenic strain of the virus (Fig. 2).

As was pointed out above, enzymic activity of the hydroxylating system depends on the state of the lipid matrix of the microsomal membranes. In particular, it was shown that modification of the microsomal membrane lipids during activation of LPO leads to a decrease in activity of the cytochrome P-450 system [5].

The quantum of fluorescent LPO products in lipid extracts of the microsomal preparations amounted (on the 5th day) to the following, calculated per microgram lipid phosphorus: 1.7 ± 0.1 (control), 1.4 ± 0.2 (apathogenic virus), and 3.5 ± 0.1 U (pathogenic virus). It must be pointed out that induction of LPO in the liver microsomes of animals infected with the pathogenic virus did not lead to any significant changes in the α-tocopherol content. In lipid extracts of membrane preparations from all three groups of animals this parameter was of the order of 0.17 ± 0.03 $\mu g/\mu g$ lipid phosphorus in each sample studied.

Activation of LPO was accompanied by structural changes in the matrix of the membranes. At 10°C the value of $2A_{//}$, calculated from the EPR spectra of 5-DS in membranes from liver tissue of mice infected with the pathogenic virus was 60.1 ± 0.8G, whereas the same parameter for membranes from tissues of the control group of animals and animals infected with the apathogenic virus was 57.8 ± 0.5 and 57.6 ± 1.0G, respectively. This is in agreement with the previously expressed view that accumulation of LPO products in lipid membranes leads to an increase in their rigidity.

Significant structural changes were found in the lipid microenvironment of the microsomal proteins in pathology. This was expressed primarily as an increase in the content of fluorescent LPO products, and a relative decrease in the content of palmitic and certain polyenic acids in the fatty acid composition of lipids covalently "cross-linked" with membrane proteins (Fig. 3).

This investigation thus revealed a decrease in the content of the catalytically active form of cytochrome P-450 and in enzymic activity of p-methyl-O-demethylase in the liver tissue of animals with the toxic form of experimental influenza. One cause of these changes may be modification of the lipid component of the microsomal hydroxylating system, due to activation of LPO.

LITERATURE CITED

1. Ya. A. Azhipa, Medico-Biological Aspects of the Use of the Electron Paramagnetic Resonance Method [in Russian], Moscow (1983), pp. 17-86.
2. N. V. Gorbunova and Kh. N. Annanepesov, Neirokhimiya, **4**, No. 3, 290 (1985).
3. N. V. Gorbunov, A. P. Volgarev, L. Yu. Taros, et al., Magnetic Resonance in Biology and Medicine [in Russian], Zvenigorod (1990), p. 16.
4. P. J. Anderson, J. Histochem. Cytochem., **15**, No. 11, 652 (1967).
5. D. Doiphin, Oxygen Radicals in Biology and Medicine, New York (1988), pp. 491-500.
6. W. L. Hubbell and H. M. McConnell, J. Am. Chem. Soc., **93**, No. 2, 314 (1971).
7. J. D. Lambeth, Phospholipids and Cell Regulation, Vol. 2, Florida (1985), pp. 190-228.

8. R. N. Masihi, W. Lange, B. Rohde-Schulz, et al., Infect. Immun., 50, 146 (1985).
9. S. C. Mogensen, Microbiol. Rev., **43**, No. 1, 1 (1979).
10. A. D. Pahimtula and P. J. O'Brien, Biochem. Biophys. Res. Commun., **62**, No. 2, 268 (1975).
11. R. I. Quigg, R. Gaines, and B. E. Wakely, Am. J. Kidney Dis., **17**, No. 3, 339 (1991).
12. V. G. Zannoni, Fundamentals of Drug Metabolism and Drug Disposition, Baltimore (1972), pp. 566-568.